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Measurement by electroantennogram of airborne pheromone in cotton treated for mating disruption of *Pectinophora gossypiella* following removal of pheromone dispensers

Uwe T. Koch[1], Wolfgang Lüder[1], Ulf Andrick[1], Robert T. Staten[2] & Ring T. Cardé[3]*

[1]*Fachbereich EIT, Technische Universität Kaiserslautern, Kaiserslautern D67653, Germany,* [2]*US Department of Agriculture, Animal Plant Health Inspection Service, 3645 Wier Avenue, Phoenix, AZ 85040, USA, and* [3]*Department of Entomology, University of California, Riverside, CA 92521, USA*

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Abstract

The presence of pheromone in cotton foliage after removal of pheromone dispensers was assessed by measuring the airborne pheromone concentration with an electroantennogram device. Plots of 0.4 ha in isolated cotton fields were treated with Shin-Etsu PBW-Rope® pheromone dispensers for mating disruption of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae). The dispensers contained (*Z*,*Z*)- and (*Z*,*E*)-7,11-hexadecadienyl acetates (gossyplure) in a 49:51 ratio and were applied at a density of 1 000/ha. The 400 pheromone dispensers were removed 1–12 days later. In four experiments involving canopy heights from 30–150 cm, the decay of the pheromone concentration was recorded repeatedly in short intervals for up to 7 h. Decay to undetectable concentrations generally occurred within 1–10 h, depending on plant size and wind conditions. In all four experiments, pheromone concentration 24 h after removal was found to be near or below detection threshold of the electroantennogram. The presence of pheromone within and above the cotton after dispenser removal would be due to re-entrainment of pheromone that had been adsorbed on cotton foliage or possibly some residual airborne pheromone.

Introduction

The pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), can be managed by application of a variety of formulations that emit synthetic pheromone over many days or even months (Staten et al., 1996). These formulations include (1) microdispersibles (beads and microcapsules), which are sprayed directly onto foliage with the same equipment used for applications of insecticide, (2) plastic hollow fibers and laminate flakes, which are applied aerially with specialized equipment and adhere to foliage with a polybutene sticker, and (3) high-dose polymer dispensers, which are hand applied (reviewed by Cardé, 2007). The density of pheromone-emitting points and their rate of pheromone emission also varies with formulation type. For the control of the pink bollworm using microcapsules, there are, for example, typically >100 000 points sources of disruptant/ha. Hollow fibers are applied aerially to achieve an average density of 10 000/ha. Polymer dispensers such as the Shin-Etsu ropes are set out by hand at 1 000/ha. The resulting airborne concentration and vertical distribution of pheromone from such formulations also varies. As measured in the field 30 cm above ground level with an electroantennogram (EAG) system (Färbert et al., 1997), the relative concentration of airborne pink bollworm pheromone from an open-ended hollow fiber (Scentry NoMate®, Scentry Biologicals, Billings, MT, USA) formulation was 10-fold less than from a sealed, hollow polyethylene tube (Shin-Etsu PBW-Rope®, Shin-Etsu, Tokyo, Japan) formulation.

The evenness of distribution of airborne disruptant throughout and above the cotton foliage is one factor that should influence the efficacy of mating disruption (Cardé et al., 1998; Cardé, 2007). In the case of the Shin-Etsu formulation, individual dispensers are placed about 3 m apart and pheromone re-entrained from foliage could aid in disruption efficacy by helping to even the distribution of

*Correspondence: Ring T. Cardé, Department of Entomology, University of California, Riverside, CA 92521, USA.
E-mail: ring.carde@ucr.edu

pheromone between the sources. Wall et al. (1981) were the first to show that airborne pheromone from artificial point sources was adsorbed onto nearby vegetation, which later emitted sufficient pheromone to induce attraction in male *Cydia nigricana* (Fabricius). The phenomenon of adsorption onto foliage and subsequent re-entrainment was later confirmed in application of synthetic pheromone formulated for mating disruption of *Epiphyas postvittana* (Walker) (Karg et al., 1994; Suckling et al., 1994). The importance of re-entrainment of pheromone from foliage to the efficacy should be relevant to high-dose formulations of disruptant that are applied as point sources in relatively low densities. The resulting distribution of pheromone therefore would be expected to be relatively heterogeneous. Residual airborne pheromone present within the canopy is a second mechanism that could account for the presence of measurable levels of pheromone away from point sources.

We used an EAG system (Sauer et al., 1992; Färbert, 1995; Färbert et al., 1997; Lüder, 1997) to measure concentrations of pheromone from Shin-Etsu PBW-Rope dispensers applied to cotton at the standard rate of one rope per 10 m^2. The overall levels present in a treated field were assessed by measuring the airborne concentration of pheromone when dispensers were present and tracking the decay in concentration in the hours following the removal of all dispensers.

The density of foliage during a growing season can vary widely. In cotton, it ranges from a few leaves per plant when the plant is 20 cm high to hundreds of leaves when the plant is 150 cm high. We evaluated two situations: cotton in early season when the plants were ca. 30 cm high and the majority of ground in the field was bare, and cotton in which plants were 60 to 150 cm high and there was little or no open space between plants in a row. The measurements reported here used an EAG measurement system to record pheromone concentration with a time resolution on the order of 10 s. Airborne adsorbent trapping methods (e.g., Caro, 1982; Flint et al., 1993) require sampling times of many hours and therefore they reveal little about the vertical distribution of pheromone and changes in concentration occurring over short time spans.

Materials and methods

Field electroantennogram system

The device used in these experiments has been described in detail in Färbert et al. (1997) and Lüder (1997). Pheromone concentration is detected by an excised antenna connected to a sensitive amplifier. The antennal holder is attached to the bottom of a vertical tube, in which a constant flow of air (14 ml/s) is maintained by a suction pump. A charcoal filter when connected to the upper tube entrance removes potentially stimulatory odors from the incoming air. The sensitivity of the antenna is calibrated by three calibration sources that are connected to the main tube to deliver puffs (0.25 ml, 0.6 s duration) of defined pheromone concentration into the main air stream. The calibration sources are 20-ml glass syringes, each holding a small open vial containing a mixture of silicone oil (Type 3, Merck no. 12525; Merck, Darmstadt, Germany) and gossyplure pheromone (ca. 95% pure, obtained from BASF, Ludwigshafen, Germany), in concentrations of 10^{-6}, 10^{-5}, and 10^{-4} ml/ml. Inside these syringes, a constant equilibrium mixture of air, oil vapor, and pheromone vapor establishes. With a stable pheromone, concentration in the calibration syringes is constant over time (Sauer, 1989); syringes are capable of delivering more than 20 000 puffs without degradation (Sauer, 1991). As shown by Kafka (1970) and confirmed by Koch et al. (2002b), the pheromone concentration in the syringes is linearly dependent on the dilution of the pheromone in the oil. Such calibration sources can be prepared any time to duplicate the identical pheromone concentrations, therefore allowing reproducible measurements of relative pheromone concentration as described below.

The antennal response amplitude A to puffs of the calibration sources is related to the pheromone concentration X in a logarithmic dose–response curve, which can be approximated by

$$A = S \log(X/X_0 + 1), \tag{1}$$

where S is the sensitivity in mV per decade and X_0 is the detection threshold of the antenna.

The measurements are structured in measurement cycles, each of 50 s duration, consisting of the calibration mode and the measurement mode. Each cycle begins with the calibration mode with the charcoal filter closed over the ambient air entrance port. Three reaction amplitudes (A_{1-3}) of the EAG to the three calibration syringes are measured. These values are used to construct the dose–response curve and to calculate S and X_0 by best fit of the data to the approximation formula (Equation 1). As these calibrations are repeated for every measurement cycle, gradual changes of the sensitivity S are known and compensated for in the calculations. As X_0 also varies with time, it is always indicated in the measurement results. The quasi-continuous calibrations permit to use several antennae for measurements over long periods and enables us to give reproducible data that permit comparison of pheromone concentrations over space and time. Usually, an antenna provides satisfactory readings for 30 min and exceptionally good antennae last for up to 100 min.

In the measurement mode, the charcoal filter is removed from the tube and outside air flows over the antenna.

Airborne volatiles can produce an EAG response, similar to a step function. As this response can be caused by background odors such as plant volatiles as well as pheromone, it alone cannot be used as a measure of pheromone concentration. When the filter is not in place and ambient air flows across the antenna, additional calibration pulses are released. The additional response to the superimposed calibration puffs on the 'background' EAG signal is used to calculate the airborne concentration of pheromone (Milli, 1993). A small additional response indicates high pheromone concentration, whereas an EAG response to the superimposed stimulus that is almost as strong as in the calibration mode indicates a very low level of ambient pheromone concentration, close to the detection threshold of the antenna.

The EAG measurement system including pumps, calibration syringes, and associated step motor drives is mounted on a compact probe. Wind velocity is measured by two thermistor anemometers, one mounted near the probe, and the other at 170 cm. Measurements were taken at probe heights of 30, 100, and 170 cm.

The amplitudes of the individual EAG reactions, as well as the wind velocity, measurement location, and temperature were stored on disk. Errors due to drift or spontaneous offsets in the EAG signal were corrected before analysis. The concentrations of pheromone were then calculated for each syringe test stimulus. Measurements (usually nine, but a minimum of three) were recorded at each probe height setting, requiring a total of approximately 3 min. The results were averaged (geometric mean). The error bars shown in the plots indicate two sources of error: (1) the standard error emerging from the calculation of the average, and (2) the error of each individual reading due to noise in the EAG signal. These two errors were added as $\sqrt{(a^2 + b^2)}$.

The pheromone concentration was originally measured in relative units. A concentration of 10^{-6} relative units is the concentration present in a calibration syringe containing one part of pheromone and 10^6 parts of oil. The pheromone concentration present in our calibration syringes was measured by gas chromatography of pheromone adsorbed onto glass capillaries from a series of syringe puffs (Koch et al., 2002a). A pheromone concentration of 10^{-6} relative units was found to be equivalent to 1.7 ± 0.15 ng m^{-3}. Thus, in the graphs the pheromone concentration is given in absolute units (ng m^{-3}).

Dispenser placement and removal

The measurements were made in cotton fields near Phoenix, AZ, USA, in 1995. The Shin-Etsu PBW-Rope dispensers are hollow polymer (polyethylene) dispensers reinforced with soft wire and PVC resin. Each contains about 78 mg of technical gossyplure, (*Z,Z*)- and (*Z,E*)-7,11-hexadecadienyl acetates of 96% purity in a 49:51 ratio (Flint et al., 1985). Dispensers were set out in cotton fields isolated by at least 5 km from other fields treated with mating disruptants. Every third cotton row received a dispenser every 3 m. A total of 400 Shin-Etsu rope dispensers were applied to 0.4 ha. The dispensers were usually fixed to the main stem of the cotton plants at a height of 10 cm (except for Experiment 4). To facilitate locating the dispensers for removal, plants carrying dispensers were marked with red cotton strings.

Dispensers were removed by 4–6 persons. Each person collected all dispensers in a row, and then sealed that row's dispensers in a plastic bag. By counting the dispensers in each bag, we ensured that no dispensers remained in the field. About 30 min was needed to remove all dispensers.

Experiment 1

Cotton plants in this field were about 30 cm high. Background measurements were made 15 June, and the dispensers set out on 16 June and removed on 17 June. Pheromone concentration was monitored for 4 h after the dispenser removal and again on 18 June and 10 July.

Experiment 2

The height of the cotton plants was about 30 cm. Dispensers were installed on 18 June and removed on 20 June. Measurements of pheromone concentration in the middle of the treated area were started shortly before the beginning of the dispenser removal, and carried out for the following 4 h.

Experiment 3

Cotton plants were approximately 60 cm high. A pre-treatment measurement was made on 16 June, dispensers were applied on 17 June, and pheromone concentration was measured 2 h after the placement of the dispensers was complete. The dispensers were removed on 22 June. During removal and the following 7 h, pheromone concentration levels were monitored continuously in the center of the treated area. The pheromone concentration was measured again on 23 June.

Experiment 4

The average plant height was about 150 cm and the dispensers were attached to the plants at 120 cm. On 13 July, pheromone concentration was measured 2 h after the installation of the dispensers. Dispensers were removed on 25 July. Pheromone concentrations were recorded continuously for a total of 7.5 h, starting 1 h before the dispenser removal, and again on 26 July.

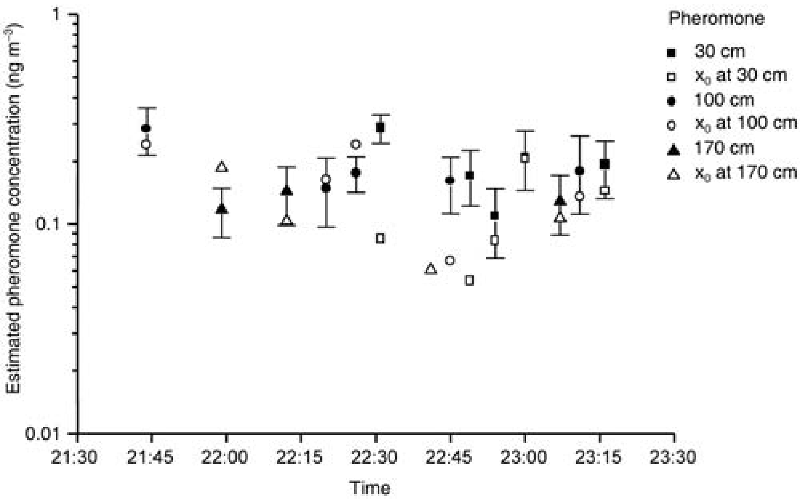

Figure 1 Experiment 1. Measurement of pheromone concentration before the application of the dispensers at probe height of 30, 100, and 170 cm. X_0 is the corresponding threshold. Almost all readings were close to threshold, indicating that no pheromone was detectable.

Results

Experiment 1

Before the placement of the dispensers in the cotton, pheromone concentration values and their confidence intervals overlapped with the values of the threshold x_o and thus there was no pheromone detectable by our measurement system (Figure 1). The pheromone levels during and after dispenser removal were measured on 17 June (Figure 2). At the beginning of removal, pheromone concentration was about 0.7 ng m^{-3} and decayed quickly to 0.14 ng m^{-3}. Beginning at 21:30 hours, the pheromone concentration measured at 30 cm increased, reaching levels exceeding those found at the beginning of these measurements. Among the factors that could account for the lower concentrations associated with elevated wind velocities are an increase in turbulence that would transport pheromone above the canopy and deplete the pheromone within the canopy and dilution of a given amount of pheromone into a larger volume of air. Pheromone was not detected on the day after removal, nor 21 days later (Figure 3).

Experiment 2

On 20 June, dispensers were removed between 19:15 and 19:30 hours (Figure 4). The pheromone concentration fell immediately until about 90 min later when it reached the threshold of our measurement system. At 19:30 and 20:10 hours pheromone concentration dropped to threshold, but returned to prior levels shortly thereafter. Measurements were taken at a height of 30 cm, as this is the

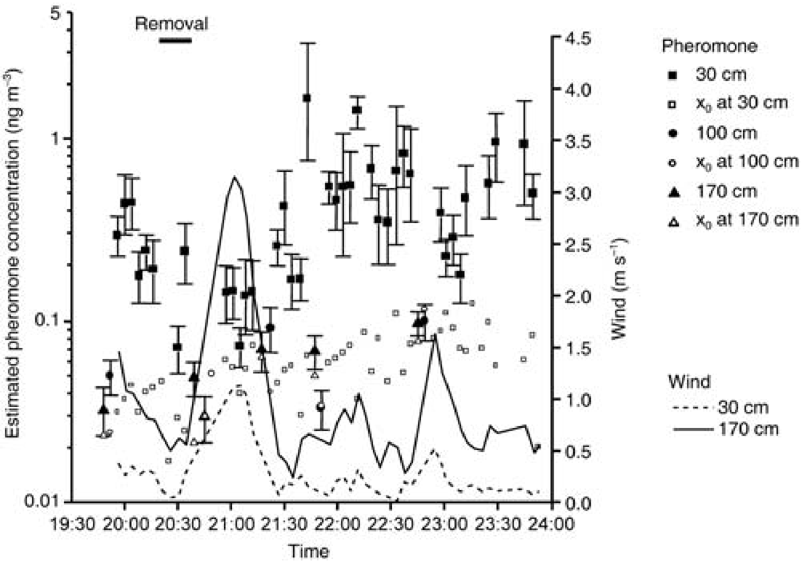

Figure 2 Experiment 1. Dispenser removal. Time of removal is indicated by a horizontal bar. Pheromone concentration measured at 30, 100, and 170 cm probe height is plotted vs. time. Pheromone concentration increased 1 h after removal of dispensers, when the wind speed dropped below 0.5 m s^{-1} at 30 cm height.

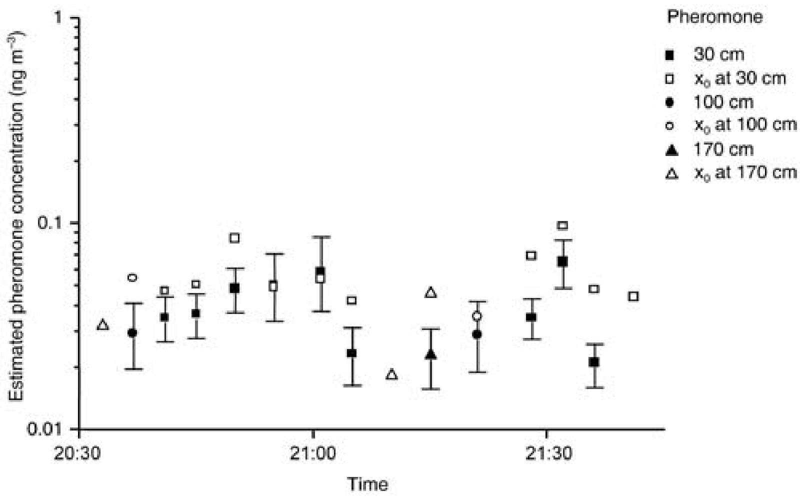

Figure 3 Experiment 1. Measurement 24 h after dispenser removal. All pheromone concentration values were near the antennal threshold of 0.03 ng m^{-3}, indicating that pheromone concentration had dropped at least 30-fold.

place where most of the changes were expected. Wind velocity was moderate during the time of removal, but increased sharply around 20:30 hours, and high wind speeds persisted for the rest of the night.

Experiment 3

Background measurements before dispenser installation were very close to the detection threshold, which ranged from 0.015–0.03 ng m^{-3}. This means that no pheromone was detected above the threshold of our measurement system. Figure 5 shows the pheromone level after dispenser removal on 22 June. A typical concentration of 1.7 ng m^{-3} was reached near ground (30 cm), while there was less pheromone at 100 and 170 cm. Both of these heights are well above the top of the canopy (60 cm), and so the lower concentrations at these heights are to be expected. Pheromone concentration levels measured eight times on the next evening from 19:20 to 20:50 hours were close to the detection threshold, that is, no airborne pheromone was detected.

Experiment 4

During dispenser removal (Figure 6), the initial pheromone concentration of 1.7 ng m^{-3} dropped to 0.3 ng m^{-3}, but after 90 min returned to near pre-removal levels (1.2 ng m^{-3}), coinciding with a decline in the wind speed to ca. 0.5 m s^{-1} at 170 cm. High pheromone concentration levels at 30 and 100 cm (within the canopy) remained 5 h after dispenser removal. In contrast, most of the pheromone concentration values measured at 170 cm were near threshold.

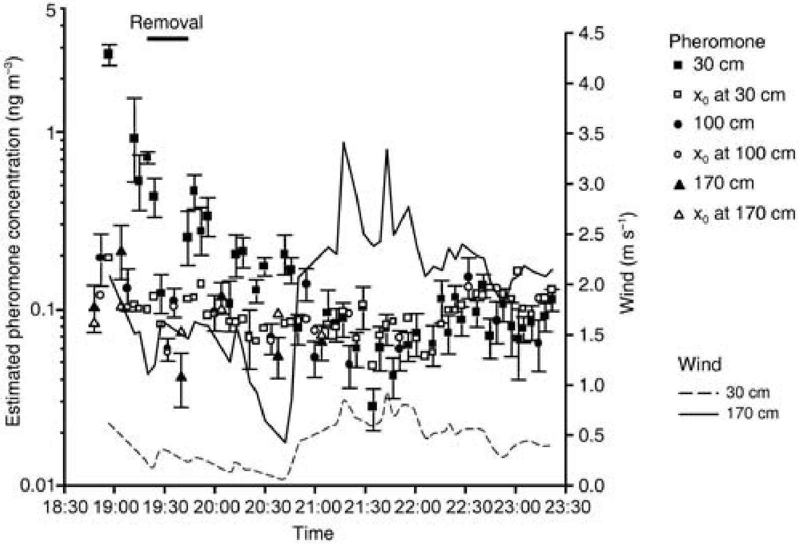

Figure 4 Experiment 2. Dispenser removal. Time of removal is indicated by a horizontal bar. In the first hour after dispenser removal, pheromone concentration declined steadily.

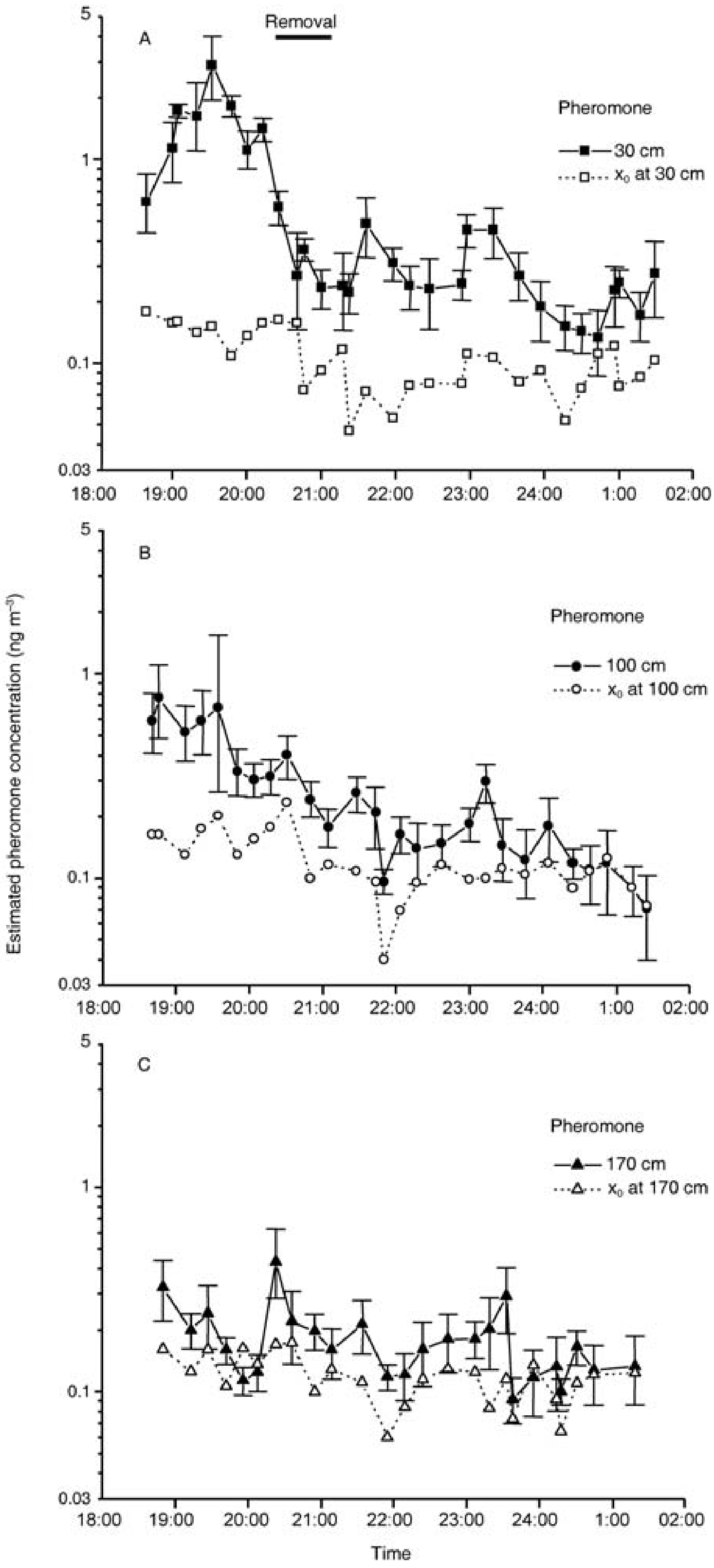

Figure 5 Experiment 3. Dispenser removal. Time of removal is indicated by a horizontal bar. Time course of the pheromone concentration measured at (A) 30 cm, (B) 100 cm, and (C) 170 cm. At 170 cm probe height, there was a much lower initial value of pheromone concentration, which continued to decline over the night.

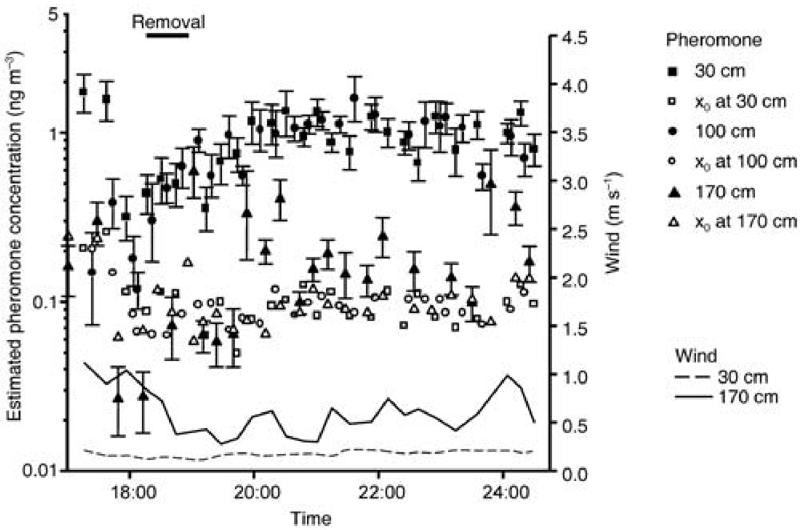

Figure 6 Experiment 4. Dispenser removal. Time of removal is indicated by a horizontal bar. Concentrations at 30 and 100 cm probe heights decayed slowly following an increase shortly after dispenser removal that corresponded to low wind speeds. In contrast, the concentration values at 170 cm were very low, although concentration often increased simultaneously with small peaks in wind speed.

Pheromone concentration measurements on the following night were between 0.1 and 0.35 ng m^{-3}, while the threshold values were between 0.09 and 0.15 ng m^{-3} (Figure 7). Particularly at 30 cm height (well within the canopy), pheromone concentration values were above threshold, indicating that a small but detectable concentration of pheromone remained 24 h after dispenser removal.

Discussion

Electroantennogram techniques

Measurements of airborne pheromone are classically made by air sampling and subsequent gas chromatography (GC) analysis (e.g., Flint et al., 1990; Bäckman, 1997). This method does not provide the time resolution required for the issues considered in our experiments.

The high sensitivity and fast response of the EAG are unique assets that have drawn investigators to its use in pheromone detection. Measurements using a system with one standard stimulus (mostly non-pheromone) as reference signal (van der Pers & Minks, 1998) have been widely used (Ryne et al., 2001; Thorpe et al., 2007). These devices yield EAG amplitudes relative to a stimulus that itself has no direct relationship to the pheromone-sensing channel, but that may represent the 'fitness' of the antenna. As a constant relationship between the reaction to the non-pheromone stimulus and the sensitivity to pheromone cannot be established with different antennae, only short-term comparisons between 'treated' and 'untreated' plots can be made, unless a large number of readings with different antennae is used, under the assumption that the sensitivity of an 'average' antenna is a reproducible quantity (e.g., Thorpe et al., 2007). Using only one reference signal cannot assign quantitative relations between two readings, because the logarithmic dose–response curve (a known property for many moth species) is not

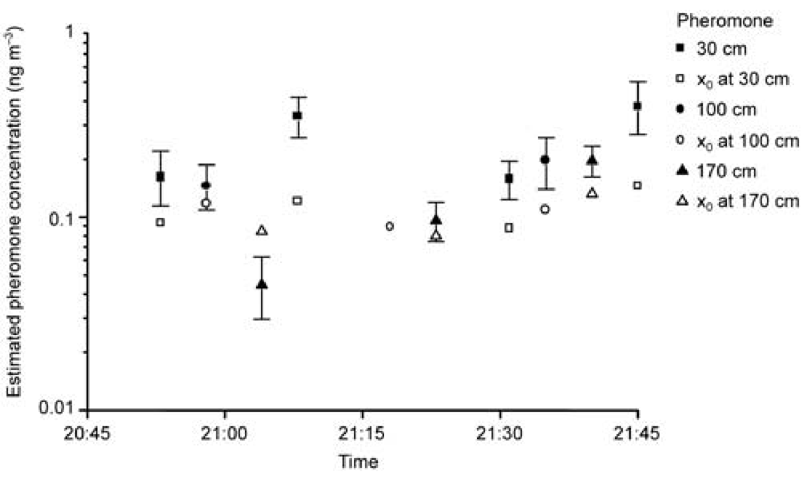

Figure 7 Experiment 4. Control measurements 24 h after dispenser removal. Only a low pheromone concentration, close to the antennal detection threshold, remained.

established and employed. In addition, these systems have no means to distinguish between EAG reactions to ambient pheromone and reactions to plant volatiles, which can generate a non-specific background signal.

In our EAG system, the sensitivity of the antenna is established in a concentration range of three decades, a range that is chosen to represent detectable atmospheric pheromone levels and permits our system to yield quantitative results that are reproducible over time. In addition to the absolute calibration for *P. gossypiella*, our system has also been calibrated for absolute concentrations of the pheromone of *Cydia pomonella* (L.) using a simultaneous measurement of air sampling/GC and EAG recordings (Bäckman, 1997; Koch et al., 1997). If there is no absolute calibration available, our system still yields relative concentration values that can be correlated with actual concentrations in the calibration syringes. Such results, although providing relative values, are independent of individual antennal properties and reproducible over time. Pheromone concentration has been measured with this system in the field with *Sesamia nonagrioides* (Lefèbvre) (Koch et al., 2002a) and *Lobesia botrana* (Denis & Schiffermüller) (Färbert, 1995; Lüder, 1997).

The problem of non-specific background from plant volatiles is overcome by the special technique of stimulus superposition over background. Examples of the successful application of this technique can be found in Koch et al. (1997) and Koch & Witzgall (2001).

Pheromone buffering

Electroantennogram measurements of concentration of pheromone following the removal of dispensers showed that concentration declines within hours by a factor of 10-fold or more. We assume that pheromone is adsorbed onto the cotton foliage when dispensers are present and that it is re-entrained after the dispensers are removed. Adsorption of a 14-carbon-chain, acetate-moiety pheromone onto foliage and subsequent desorption has been shown in apple orchards (Karg et al., 1994; Suckling et al., 1994). However, we note that some of the pheromone detected following dispenser removal may be attributable to residual airborne pheromone within the plant canopy that has been redistributed by turbulent diffusion rather than by re-entrainment from foliage.

In the current measurements in cotton, the maximum pheromone concentrations measured when formulation was present were about 1.7 ng m^{-3} (Figures 2 and 4–6). This matches the 12-h-long night time (18:00–06:00 hours) adsorbent samples of Flint et al. (1993). Their estimates of pheromone concentration of pink bollworm pheromone in cotton using the same Shin-Etsu formulation 20 days post-application ranged between 1–2 ng m^{-3} at 15 and 40 cm sampling heights, with a canopy height of 40 cm at the time of the measurement. Flint et al. (1990) also suggested that 1.3 ng m^{-3} is the minimum concentration for this formulation to be effective in mating disruption.

When wind velocity was low, pheromone tended to remain within the canopy and caused a persistence of moderate to high levels of pheromone for several hours. Not unexpectedly, the decay in concentration was most rapid with increased wind velocities. The decay time in these measurements ranged from about 30 min (Figure 4) to an estimated 10 h (Figure 6). The extent to which the presence of pheromone following dispenser removal was due to re-entrainment of pheromone from cotton or to residual airborne pheromone within the cotton canopy cannot be determined from these measurements, but it is likely that airborne pheromone within the cotton canopy would either be adsorbed onto the leaves or quickly dispersed out of the canopy by air movement. A simple model, however, can be used to calculate a lower limit for the decay time of pheromone concentration in the absence of any storage or buffering effects of foliage or soil. At a deployment of 1 000 dispensers/ha and an assumed canopy height of 1 m, each dispenser is surrounded by a 'working air volume' of 10 m^3. At a typically measured steady state pheromone concentration of 1 ng m^{-3}, the working air volume must contain about 10 ng of pheromone (not considering any pheromone stored on foliage). Because a typical release rate of a PBW-Rope dispenser is in the order of 1 mg day^{-1} = 11.6 ng s^{-1} (Flint et al., 1990), this means that the working air volume would be filled up with pheromone with a time constant of roughly 1 s.

In a steady state, release rate and depletion mechanisms are in equilibrium, which indicates that the depletion rate is on the order of 11 ng s^{-1}. When the dispensers are removed, the working air volume should be depleted of pheromone with a time constant of roughly 1 s, if no other effects of storage or buffering in the foliage or in the soil are present. Obviously, the observed decay time constants are several orders of magnitude higher, indicating considerable storage and buffering effects. Regardless, the relatively homogeneous distribution of airborne pheromone found in these measurements should enhance the efficacy of low-concentration, high-dose dispensers by ensuring that males within the cotton foliage encounter at least a moderate concentration of pheromone, even if they are several meters away from the nearest point source of disruptant.

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